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14. ABSTRACT

We have derived cardiomyocytes (heart cells) with high purity using an optimized approach that can coax human induced pluripotent stem cells (hiPSCs) into heart cells in combination with a two-day lactate selection method that can remove non-cardiomyocytes from the culture. With the availability of robust hiPSC-derived cardiomyocytes (hiPSC-CMs), we have compared different supporting scaffolds and established a highly effective system that enables robust pulsatile tissue formation. Specifically, a novel approach for developing robustly contractile tissue constructs was established by seeding hiPSC-CMs onto a laser-cut, thin sections of decellularized porcine myocardium that enable the alignment of the seeded cells along native collagen fiber for efficient cell-cell contact and gap junction formation. Moreover, human cardiac fibroblasts have been shown to improve the even distribution of hiPSC-CMs in the tissue constructs and to enhance the contractile force. Furthermore, decellularized human umbilical arteries (HUAs), readily available and mechanically robust tubular scaffolds that can endure high blood pressure in humans, have been successfully developed and utilized for wrapping the pulsatile tissue constructs with the goal of generating tubular pulsatile conduits. We anticipate that novel and effective tissue-engineered pulsatile conduits will be established for comprehensive functional studies in vitro and in vivo in the coming research period.

15. SUBJECT TERMS
Human induced pluripotent stem cells, cardiomyocytes, contractile force

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1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The primary objective of this proposal is to establish tissue-engineered pulsatile conduits (TEPCs) using cardiomyocytes (CMs) derived from human induced pluripotent stem cells (hiPSCs) for surgical correction for patients with single ventricle cardiac anomalies that afflicts approximately 1 in 1000 live births. TEPCs will be transplanted into the inferior vena cava of nude rats in order to mimic the clinical setting of single ventricle disease, and whether TEPCs can develop into functional pulsatile conduits will be investigated. Establishment of TEPCs using hiPSC-derived CMs will set the stage for the development of autologous tissue engineered pulsatile conduits for clinical intervention in single ventricle patients.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Tissue-engineered pulsatile conduits, human induced pluripotent stem cells, cardiomyocytes

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

The major goals of the project in the reporting period were to further optimize the cardiac differentiation of human induced pluripotent stem cells (hiPSCs) towards cardiomyocytes (CMs), and to develop a more robust approach to generate CMs with high purity with shorter period of lactate selection to ensure the production of heathier CMs. Moreover, we would like to establish the most effective system in which CMs interact with the optimized supporting matrix or biomaterials and generate the most robust contractile force. Furthermore, we worked with Yale IACUC and DOD Animal Care and Use Review Office and planned to get approval for animal protocols in the reporting period.

DOD Animal Care and Use Review Office approval was granted on December 20, 2016. We initially planned to do karyotype and pluripotency assays for hiPSC-CMs at the beginning of the project, and then elected to first further optimize the efficiency of CM derivation and enrichment approach. In the original application, a seven-day lactate selection was used to highly enrich CM from the differentiated culture due to a suboptimal hiPSC cardiac differentiation efficiency. With an optimized approach to enhance cardiac differentiation (details see the section below), we could derive CMs with high purity with a two-day lactate selection approach recently (May 2017). This is a very significant progress and can result in functionally robust CMs since a greatly shortened lactate selection reduces metabolic stress triggered by long duration of lactate culture. The karyotyping and pluripotency tests for hiPSC-CMs and CM-supporting cells such as human cardiac fibroblasts and human foreskin fibroblasts (both commercially available) will be performed in the coming research period.

With the availability of robust hiPSC-CMs, we have recently established a highly effective system that supports robust CM contractile force by comparing and contrasting seeding CMs onto different supporting matrix (details see the sect ion below) (experimental period: starting

from last year till June 2017). With the optimized hiPSC-CMs and the robust supporting matrix, we expect to see important research progress in generating hiPSC-CM-based tissue-engineered pulsatile conduits with strong contractile force in the coming research period.

What was accomplished under these goals?
For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

1. Major Activities

I was an invited speaker at Yale Seminar Series in Biomedical Research, Department of Internal Medicine; at the Cardiovascular Biology Seminar Series, Department of Medicine, Emory University in February 2017; at Cardiovascular Institute (CVI), Stanford University in April 2017; at Columbia University Department of Pharmacology Seminar Series in June 2017; and at Center for Translational Medicine and Department of Pharmacology Seminar Series at Temple University in June 2017. I also gave a talk at Boston International Society of Stem Cell Research (ISSCR) Annual meeting in June 2017. Additionally, I attended AHA Scientific Sessions at New Orleans in November 2016, Stanford Drug Discovery Conference 2017, and StemConn 2017. I reviewed manuscripts for Cell Report, Acta Biomaterialia, Current Opinion in Biomedical Engineering, Scientific Reports and AHA Statements. I also reviewed two NHLBI PPGs, NHLBI CCHF study section and British Heart Foundation grants. My group had biweekly joint meetings on engineered cardiovascular tissue with Drs. Laura Niklason and Stuart Campbell, and cardiac physiology with Dr. Lawrence Young. I also attended weekly Yale cardiology faculty meeting, the bi-weekly Yale Cardiology Grand Round and Yale VBT Program Seminar Series, the monthly Yale Stem Cell Center Research Forum, and the annual retreat of Yale VBT Program and Yale Stem Cell Center. I have been directing Yale Stem Cell Research Forum for the past seven years. I continued to teach a class for Yale Stem Cell Center (GENE 655). Furthermore, I mentored three postdocs to write Connecticut Stem Cell grants and one postdoc to write Yale Brown-Coxe fellowship.

2. Specific Objectives

In this research period, we aimed at further enhancing the cardiac differentiation efficiency of hiPSCs into cardiomyocytes. In addition, we tried to achieve a shorter duration of lactate selection in order to generate CMs with high purity and less metabolic stress. Furthermore, we aimed at establishing a robust type of supporting matrix or biomaterial that interact with CMs to develop strong contractile force.

3. Significant Results or Key Outcomes

3.1. Derive highly enrich hiPSC-CMs.

The PI's group previously performed cardiac differentiation by an early activation of Wnt signaling with glycogen synthase kinase 3 inhibitor CHIR99021 and a subsequent inhibition of Wnt signaling with IWP4. The PI's group then used a glucose-depleted DMEM medium

containing 4mM lactate to enrich cardiomyocytes by negatively selecting against non-cardiomyocytes in seven days, since non-cardiomyocytes cannot grow effectively in lactate-containing medium. However, a seven-day lactate selection may negatively affect hiPSC-CM function due to a prolonged metabolic stress. We thus wished to address this challenge. It has been known that low dose of CHIR99021 leads to suboptimal production of mesodermal precursor cells and inefficient cardiac differentiation, while high dose of CHIR99021 results in cellular toxicity. In this research period, we have established a highly enhanced cardiac differentiation approach by including CHIR99021 at 20 μM and improving cell viability due to ChIR99021 toxicity by including 25% (volume) of stem cell medium mTESR in the differentiation medium. This approach has allowed us to use glucose-depleted DMEM media containing 4mM lactate to highly enrich cardiomyocytes by negatively selecting against non-cardiomyocytes in two days. The availability of a robust production of functionally enhanced hiPSC-CMs allows us to optimize the conditions for robust production of tissue-engineered pulsatile conduits.

3.2. Tissue-engineered pulsatile constructs based on polyglycolic acid (PGA) biodegradable and collagen type I

Two biomimetic scaffold materials such as polyglycolic acid (PGA) and rat tail collagen type I were used to establish tissue-engineered pulsatile constructs with CMs derived from human induced pluripotent stem cells (hiPSCs). Beating CMs (Figure 1A) were seeded on PGA or mixed with collagen gel on the mold to form a ring shape and cultured for two weeks (Figure 1B and C). CMs seeded onto PGA scaffold appeared to be moderately contractile, while CM tissue rings formed with collagen gel showed stronger contractile force, suggesting that collagen matrix may help hiPSC-CMs to more effectively form cell-cell contact that is required for contractility.

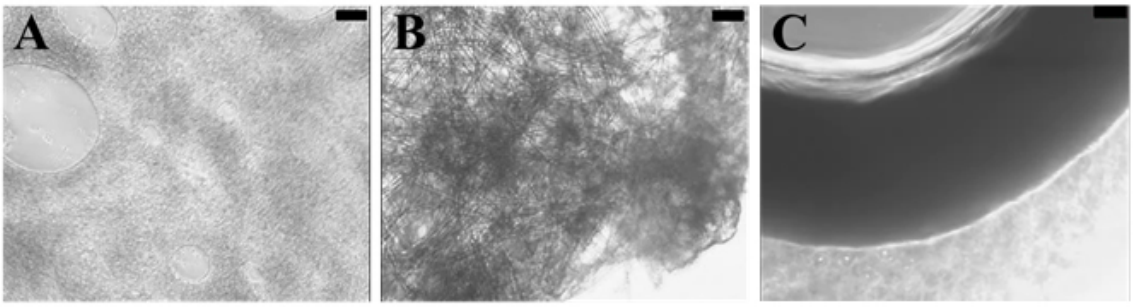


Figure 1. Biomimetic approaches for contractile heart tissue system using day 14 differentiated human iPSC derived cardiomyocytes (A). (B) 5x5mm PGA was incubated with 1ml of 1M NaOH for 1min and washed with water three times and then dried for 15 min. 0.7 million CMs were seeded onto dried PGA, and cultured for two weeks. (C) Polydimethylsiloxane (PDMS) mold was used to cast wells with 2% agarose solution in DMEM. The agarose molds were equilibrated in RPMI/B27 with 10% FBS overnight. To fabricate the ring-shape tissue, 1.2 million CMs on day 14 were mixed in 33ul of gel mixture and 6.4ul of RPMI/B27 and added into the wells in agarose mold. After 1 hour, 5ml of medium was added to each well. The medium was changed every other day, and the rings were cultured for 14 days after seeding the cells.

CMs in heart interact with endothelial cells (EC) and cardiac fibroblasts (CF). CF modulates the cardiac ECM and provide structural integrity to the heart, while EC affects cardiac metabolic activity and maturation. We next investigated the effect of CF and EC for CM remodeling using tissue rings mixed with CMs and collagen gel (Figure 2). As the arrows showed, distribution of

CMs was not even in the tissue rings containing CM only or CM plus EC. In contrast, CMs co-cultured with CF or CF plus EC showed an even distribution in tissue rings, suggesting that CF may provide a more favorable environment for CM to migrate and form more homogenous tissue.

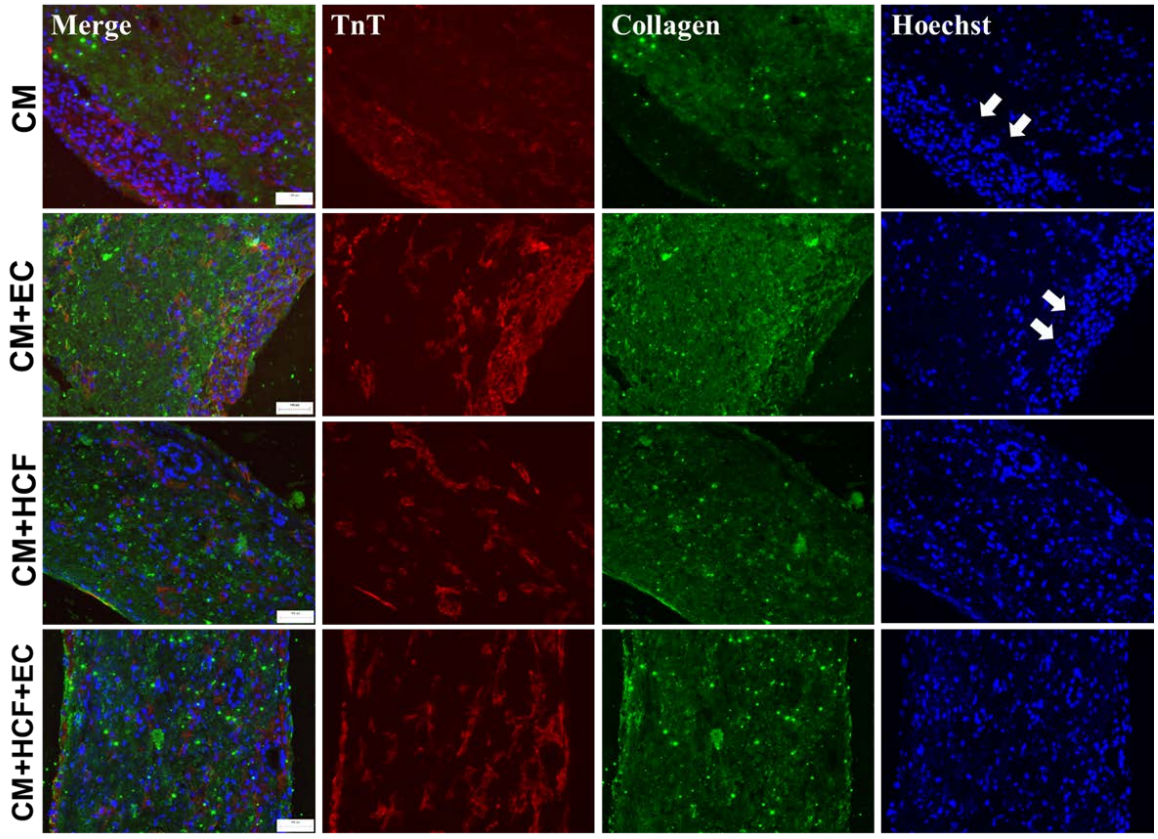


Figure 2. Histological comparison of collagen gel rings derived from composition of three types of cells, such as iPSC-derived cardiomyocytes (CM), human umbilical vein endothelial cells (HUVEC) and human cardiac fibroblasts (HCF). CM: 1.2 million cells; CM+EC: 0.96+0.24million cells; CM+HCF: 0.96+0.24million cells; CM+HCF+EC: 0.96+0.12+0.12million cells. Both HUVEC and HCF are commercially available. Size bar = 100um.

3.3. Robust generation of tissue-engineered pulsatile constructs based on decelluarized porcine heart matrix

It's an encouraging progress that tissue rings created by mixing iPSC-CMs with a collagen gel matrix generated an improved contractile force compared with tissues derived from iPSC-CMs seeded on PGA scaffolds. Notably, collagen-gel based CM tissues did not generate robust contractile force reproducibly from batch to batch, potential due to stochastic cell-cell junction formation in the tissue construct. Hence, an ideal system for producing consistent contractile force would be a test specimen containing both cells and matrix in their native arrangement. We have developed a novel approach for deriving robust contractile tissue constructs by seeding hiPSC-CMs into a laser-cut scaffold made from thin sections of decellularized porcine myocardium (Fig. 3A). Such tissue constructs are thin (<150μm) and thus allow oxygen delivery and light microscopy. Furthermore, alignment of the cells into cardiac fibers resembling those seen in vivo would allow highly efficient cell-cell contact and gap junction formation. Our

scaffolds provide realistic conditions for tissue assembly, possessing native extracellular matrix components and structure, and the resulting tissue constructs are robustly contractile (Figure 3B, 4 and 5) and exhibit physiological characteristics that mimic intact adult myocardial preparations.

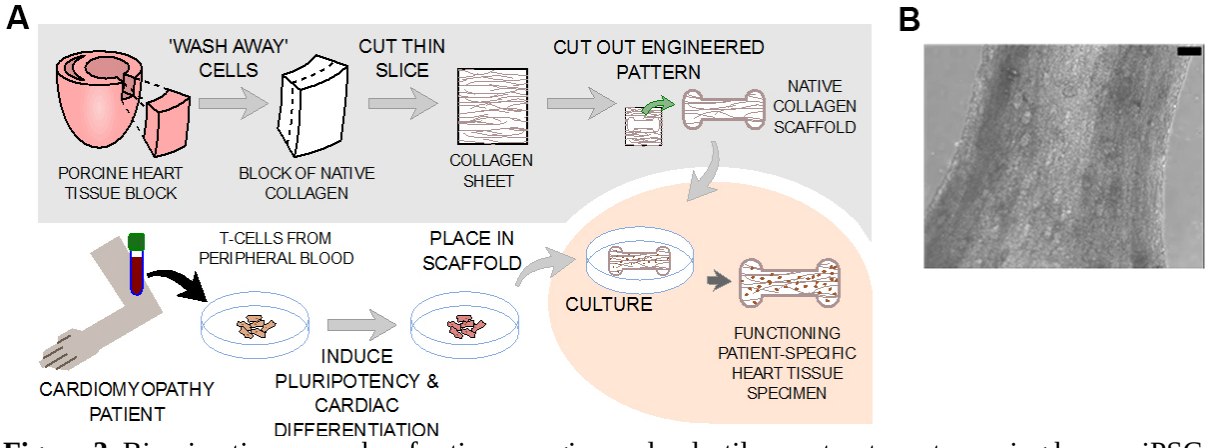


Figure 3. Biomimetic approaches for tissue-engineered pulsatile constructs system using human iPSC derived cardiomyocytes (A). (B) Laser-cut porcine heart tissues were placed into both ends of the culture frames and decellularized with 0.5% SDS solution for 40 min (porcine tissues are commercially available). Decellularized tissues were washed with PBS three times and placed with 50ul of 10% fetal bovine serum (FBS)-containing medium in the seeding wells. 75ul of medium with 1 million cells were seeded onto the construct evenly. After 2 hours, 500ul of DMEM containing 10% FBS medium was added and cultured for two days. The medium was changed every other day with RPMI/B27 and were cultured for 14 days before harvesting. Size bar = 100um

We next examined the distribution of CMs with H&E staining of paraffin sections of the pulsatile tissue constructs made from three types of cell compositions: purified CMs, purified CMs with HCF, and purified CMs with human foreskin fibroblasts (HFF) (Figure 4). Both HCF and HFF significantly improve the even distribution of CMs in the tissue constructs.

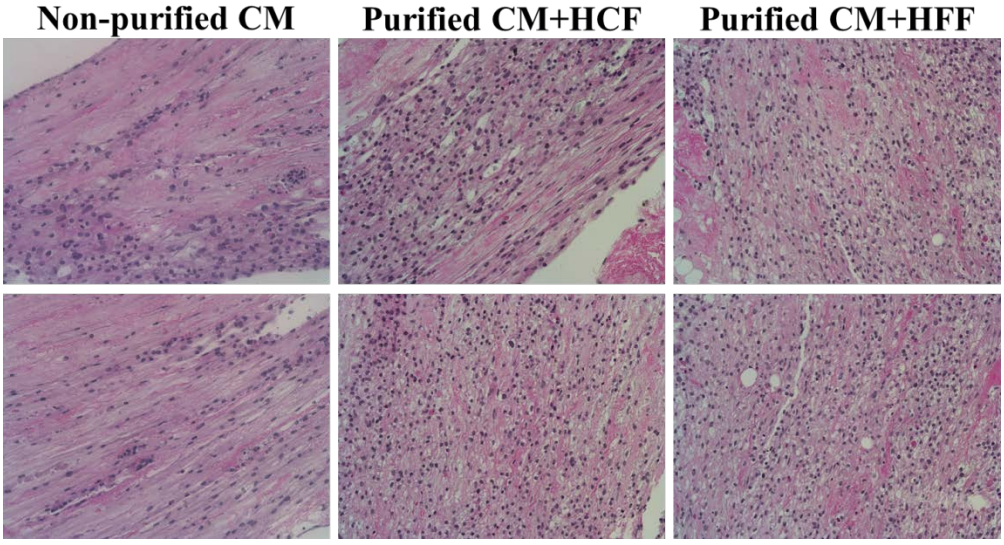


Figure 4. Histological comparison of pulsatile tissue constructs derived from composition of three types of cells: Non-purified CMs (1million); purified CMs (0.7 million) with HCF (0.3 million); and purified

CMs (0.7 million) with HFF (0.3 million). Both HFF and HCF are commercially available. Each EHTs were stained with H&E. Tissue sections were incubated with primary antibodies diluted in blocking solution (5% normal goat serum in PBS) overnight at 4°C in a humidified chamber. Sections were washed three times with Tris-buffered saline, incubated with appropriate secondary antibodies diluted 1:500 in blocking solution for 1 hour at room temperature, and washed again three times. Images were acquired using a Zeiss microscope and captured using Velocity software. For hematoxylin and eosin staining, the tissue samples were paraffin embedded and cut into section of 5μm by Yale Pathology Tissue Services based on standard protocol.

As shown in Figure 5, we next analyzed peak tension, cross sectional area (CSA) and peak tension normalized by cross-sectional area at a defined length (1.05 of culture length) and frequency (1Hz) for three type's pulsatile tissue constructs including cardiomyocytes (CMs), CMs with human cardiac fibroblasts (HCF) and CMs with human foreskin fibroblasts (HFF). Tissue constructs made from CMs mixed with HCF showed a strong trend of elevated contractility and consistent remodeling, compared to those made from sole CMs and CMs cocultured with HFF. Thus, we have established a novel approach and promising cell compositions to make tissue constructs with strong contractility, setting the foundation for developing robust tissue-engineered pulsatile conduits in the coming research period.

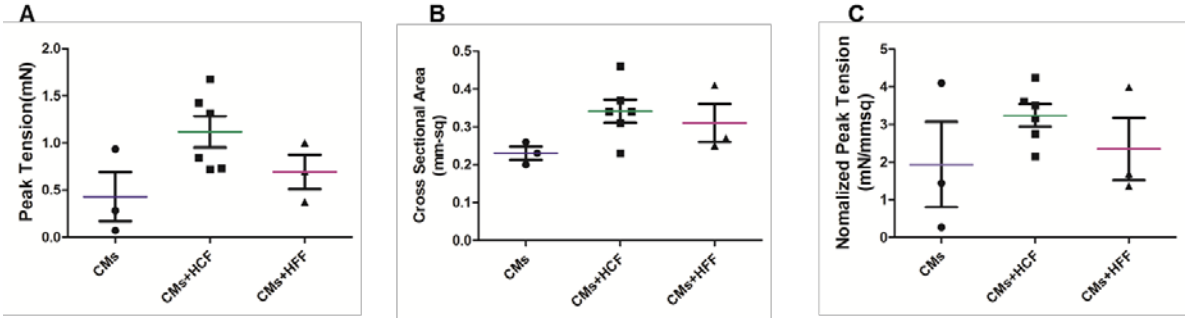


Figure 5. Contractile function and cross sectional area (CSA) of three type's pulsatile tissue constructs including cardiomyocytes (CMs), CMs with human cardiac fibroblasts (HCF) and CMs with human foreskin fibroblasts (HFF). The tissue constructs were immersed in a temperature-controlled perfusion bath equipped with electrodes for field stimulus. Throughout measurements, scaffolds were perfused with freshly oxygenated Tyrode's solution (in mM: NaCl 140, KCl 5.4, MgCl2 1, HEPES 25, glucose 10, and CaCl 1.8; pH adjusted to 7.35). Peak tension was normalized by cross-sectional area at a defined length (1.05 of culture length) and frequency (1Hz).

3.4. Establishing conditions for making tubular conduit constructs using decellularized human umbilical artery

After establishing a robust approach to generate highly contractile tissue constructs, we next tried to develop a tubular tissue construct in order to wrap the contractile tissue around to generate tissue-engineered pulsatile conduits for potential clinical application in treating single ventricle heart defect. Decellularized human umbilical artery (HUA) is being used as the scaffold to generate tubular conduits Figure 6A, since it is readily available and mechanically sound to support blood pressure of vena cava in humans. We tested burst pressure for the decellularized HUA, and the measurement came out to about 780mmHg. We next tested gelatin glue with native pig heart sheets (150um thick) for adherence to decellularized HUA, and images were taken after shaking in PBS. Magnified image of heart sheet adhered to HUA with gelatin glue were taken and as shown in Figure 6B. We next measured outer diameter changes of decellularized HUA at rest and under 40mmHg luminal pressure. Outer diameter was measured

using imageJ and found to be 0.341cm at rest (Figure 6C) and 0.439cm at 40mmHg (Figure 6D). Thus, we have established an effective approach to wrap tissue construct around decellularized HUA. Additionally, the measurement of outer diameter of HUA under flow will help us to effectively design dimensions of molds for the generation of custom pulsatile, engineered heart tissues to wrap the tubular scaffold of the beating conduit.

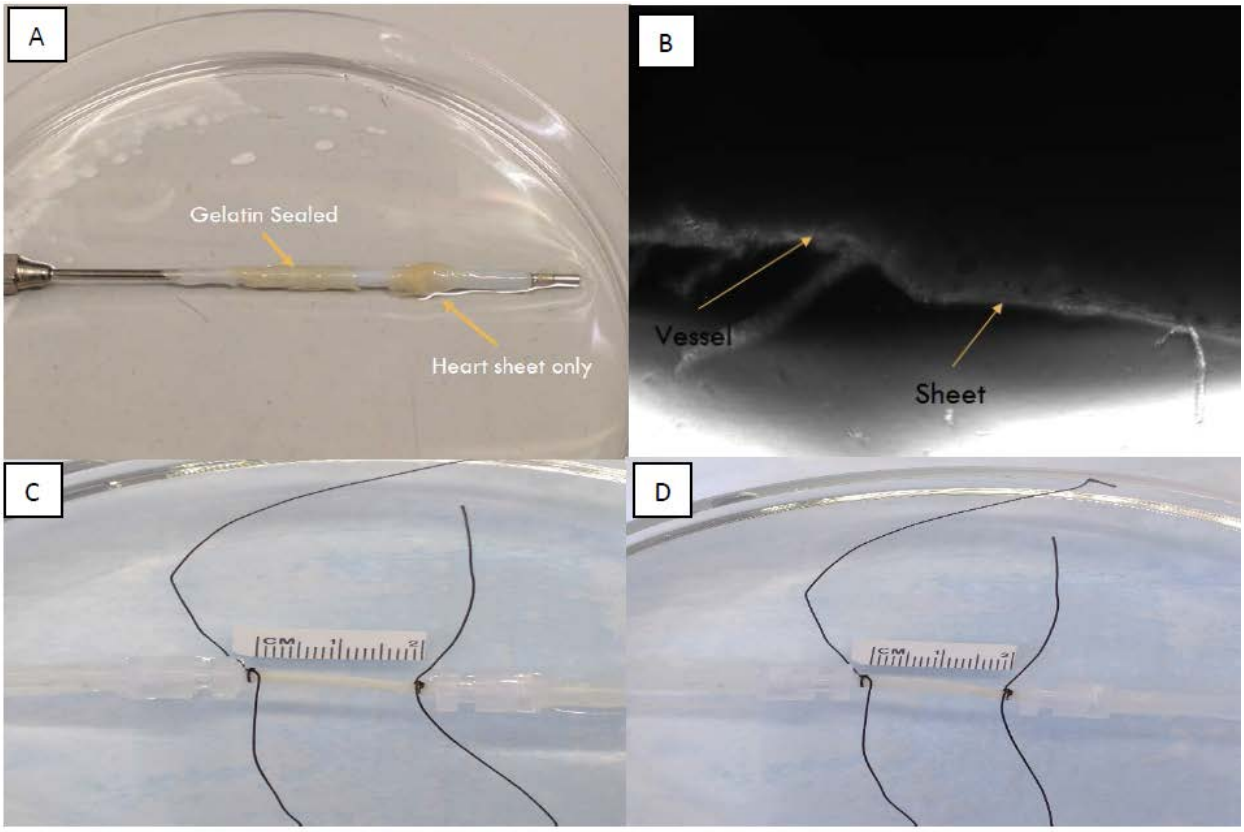


Figure 6. Development and characterization of human umbilical artery scaffold. The anonymous umbilical cords were taken from Yale New Haven hospital within HIPAA regulations and dissected to obtain two human umbilical arteries per chord. The freshly removed arteries were cleaned of remaining Wharton's jelly. PBS was flowed through the lumen of cleaned human umbilical arteries to remove any blood clots. Chords were treated with CHAPS buffer for 24h at 37^0C. Thorough PBS washes were done to remove the buffer before treating with 10% SDS for 24h a 37^0C. Arteries were washed with PBS and treated with 20% FBS diluted in PBS for 24h at 37^0C to remove any remaining nuclei. The FBS solution was washed thoroughly after treatment. All steps were performed in sterile conditions. Details (A-D) please see the texts above.

Molds for polydimethylsiloxane (PDMS) seeding (Figure 7A) and wrapping (Figure 7B) chamber. Adapting design from the collaborating Campbell lab, the mold design allows for generation of custom 14.5mmx1cm engineered heart tissues to wrap the tubular scaffold of the beating conduit. Decellularized pig heart matrix will be seeded with iPSC derived cardiomyocytes within these chambers and allowed 48h to infiltrate the tissue. The wrapping chamber will be utilized to minimize handling stress and possible damage to the engineered heart tissue when wrapping around the tubular vessel scaffold. Indents in the mold design will create harnesses to keep cannulated vessel scaffold and engineered heart tissue in place during wrapping procedures. The wrapping chamber is also capable of holding media to prevent drying

of tissue during wrapping and temporary maturation of the beating conduit before moving to flow bioreactor for further maturation. With the availability of robustly contractile engineered tissues based on hiPSC-CMs and the mechanically sound decellularized HUA as the supporting scaffolds, we expect to establish novel and effective tissue-engineered pulsatile conduits for extensive functional studies in the coming research period.

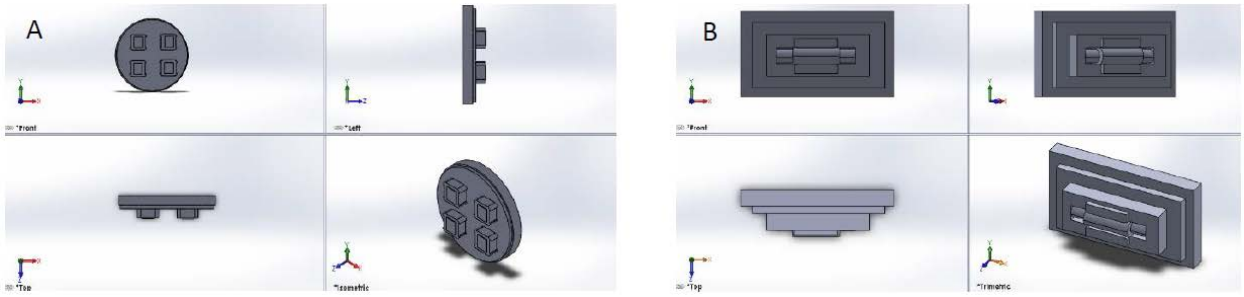


Figure 7. Designing of molds for PDMS seeding (A) and wrapping(B) chamber. Details please see texts above.

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Dr. Jinkyu Park has significantly expanded his career from stem cell biology into cardiac tissue engineering. Specially, he has learned how to make collagen gel-based heart tissue engineering, as well as generating pulsatile tissue constructs by seeding human iPSC-derived cardiomyocytes into decellularized porcine heart matrix. In addition, Dr. Liqiong Gui has expanded her career from vascular tissue engineering into cardiac tissue engineering. Specially, she has worked with Jinkyu and developed collagen gel-based heart tissue engineering. Finally, Christopher Anderson, a graduate student, has learned how to culture human iPSC, perform cardiac differentiation, developed decellularized HUA as scaffold to support pulsatile tissue construct, and designed molds for making pulsatile beating conduits.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of

these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Yibing was invited to give a talk at American Heart Association, and presented the approach of the pulsatile tissue constructs in Portland, Oregon on July 12, 2017.

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We have established an effective approach to further improve the yield of cardiomyocyte (CM) derivation from hiPSCs (hiPSC-CMs) and to achieve a shorter duration of lactate selection for the generation of cardiomyocytes with high purity and less metabolic stress. Also, human cardiac fibroblasts (HCF) may enhance the contractility of hiPSC-CMs. We will perform karyotyping and pluripotency tests for hiPSC-CMs and HCF in the coming research period. Our current studies indicate that HCF may enhance contractility of pulsatile tissue constructs based on hiPSC-CMs seeded onto decellularized porcine heart matrix. We will further confirm this result by increasing sample number and also optimize culture medium to achieve optimal contractility. With the availability of PDMS seeding mold and wrapping mold, we will generate custom 14.5mmx1cm engineered heart tissues for wrapping the tubular scaffold of the decellularized human umbilical artery (HUA) in order to develop tissue-engineered pulsatile conduits (TEPCs). We will consider electrical pacing and/or cyclic stretching for enhancing contractility of TEPCs in the coming research period. Pressure development of TEPCs will be measured, and histological analyses will be performed to examine cardiac protein expression. After developing effective TEPCs, we will investigate the function of TEPCs in a rat venous interposition graft model, followed by ultrasound studies for pulsatility and extensive histological studies for cardiomyocyte marker expression, survival and proliferation.

We will continue our collaboration with Drs. Stuart Campbell and Lawrence Young and attend the joint cardiovascular tissue engineering and physiology meetings. I will also join Yale Cardiology Grand Round and Yale VBT Seminar Series, the Yale Cardiology Faculty meeting, the Yale Cardiology/VBT annual retreat, the Yale Stem Cell Center annual retreat, the Yale Stem Cell Center Monthly Research Forum Series. In order to enhance collaborations and scientific communications, I plan to attend scientific meetings organized by American Heart Association, Department of Defense and others. I will continue to direct the monthly Yale Stem Cell Research Forum for scientific collaboration among Yale stem cell scientists. I will keep contributing to scientific community by reviewing research proposals and manuscripts. We plan to submit one manuscript based on the research project supported by DOD. Additionally, we plan to submit one grant proposal to further expand this exciting beating conduit project in the coming research period.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

We have established novel laser-cut scaffolds derived from thin sections of decellularized porcine myocardium, seeded hiPSC-CMs onto these scaffolds, and successfully developed robust, contractile tissue constructs. Importantly, the alignment of the hiPSC-CMs along native cardiac collagen fibers allow highly efficient cell contact and gap junction formation, leading to robust pulsatile tissue formation. Furthermore, decellularized human umbilical artery (HUA) has provided readily available, excellent tubular scaffold for wrapping engineered pulsatile tissues in order to develop tissue-engineered pulsatile conduits. Thus, the current study has made a significant impact in the field of engineering pulsatile conduits for patients with single ventricle heart defect.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

The success of developing engineered pulsatile tissue constructs using human iPSC-derived cardiomyocytes and decellularized porcine heart matrix will likely inspire the development of other engineered tissue constructs using iPSC differentiation and decellularized organ matrix in the near future.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report.

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

We have made a modest change in generating pulsatile tissue constructs by seeding human iPSC-derived cardiomyocytes onto porcine decellularized heart matrix instead of polyglycolic acid (PGA) scaffolds, since decellularized heart matrix supports cell contact and contractility much more effectively than PGA scaffolds.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Nothing to Report.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals.

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Anderson CW, Boardman N, Luo J, Park J, Qyang Y. Stem Cells in Cardiovascular Medicine: the Road to Regenerative Therapies. Current cardiology reports. 2017 April;19(4):34. PubMed PMID: 28324469.

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

  Nothing to Report.

Other publications, conference papers, and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Yibing gave a talk and presented the approach of the pulsatile tissue constructs at American Heart Association Annual Basic Science meeting in Portland, Oregon on July 12, 2017.

- **Website(s) or other Internet site(s)**
  List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report.

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

Nothing to Report.

- **Inventions, patent applications, and/or licenses**
  Identify inventions, patent applications with date, and/or licenses that have resulted from the research. State whether an application is provisional or non-provisional and indicate the application number. Submission of this information as part of an interim research

performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report.

- **Other Products**

  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment, and/or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

  - *data or databases;*
  - *biospecimen collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source

of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Name:	Yibing Qyang, Ph.D.
Project Role:	PI
Nearest person month worked:	1.0
Contribution to Project:	Dr. Qyang leads the project as the principal investigator and is responsible for the general management and development of this proposed study
Name:	Jinkyu Park, Ph.D.
Project Role:	Other personnel (Postdoctoral scientist)
Nearest person month worked:	8.0
Contribution to Project:	Dr. Park will establish the production of functional ventricular cardiomyocytes (VCMs) from human induced pluripotent stem cells (hiPSCs) and collaborate with Dr. Niklason's group to generate tissue-engineered pulsatile conduits (TEPCs).
Name:	Liqiong Gui, Ph.D.
Project Role:	Other personnel (Collaborator)
Nearest person month worked:	2.0
Contribution to Project:	Dr. Gui will collaborate with Dr. Park and establish TEPCs using VCMs derived from hiPSCs by providing tissue-engineering expertise.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.
Provide the following information for each partnership:
Organization Name:
Location of Organization: (if foreign location list country)
Partner's contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner's facilities for project activities);*
- *Collaboration (e.g., partner's staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner's staff use each other's facilities, work at each other's site); and*
- *Other.*

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

Not applicable.

COLLABORATIVE AWARDS: For collaborative awards, independent reports are required from BOTH the Initiating PI and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to **https://ers.amedd.army.mil** for each unique award.

QUAD CHARTS: If applicable, the Quad Chart (available on https://www.usamraa.army.mil) should be updated and submitted with attachments.

9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

Please see the original copy of a published peer-reviewed article following page 21.

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REGENERATIVE MEDICINE (SM WU, SECTION EDITOR)

Stem Cells in Cardiovascular Medicine: the Road to Regenerative Therapies

Christopher W. Anderson [1,2] · Nicole Boardman [1,3] · Jiesi Luo [1,3] · Jinkyu Park [1,3] · Yibing Qyang [1,3,4,5]

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Abstract
Purpose of Review The purpose of this review is to provide a broad overview of current trends in stem cell research and its applications in cardiovascular medicine. Researches on different stem cell sources, their inherent characteristics, and the limitations they have in medical applications are discussed. Additionally, uses of stem cells for both modeling and treating cardiovascular disease are discussed, taking note of the obstacles these engineered interventions must overcome to be clinically viable.
Recent Findings Tissue engineering aims to replace dysfunctional tissues with engineered constructs. Stem cell technologies have been a great enabling factor in working toward this goal.
Summary Many tissue-engineered products are in development that utilize stem cell technology. Although promising, some refinement must be made to these constructs with respect to safety and functionality. A deeper understanding of basic differentiation and tissue developmental mechanisms is required to allow these engineered tissues to be translated into the clinic.

Keywords Stem cells · Cardiovascular · Regenerative medicine · Induced pluripotent stem cells · Embryonic stem cells · Tissue engineering

This article is part of the Topical Collection on *Regenerative Medicine*

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Introduction

Cardiovascular disease is globally the leading cause of mortality with an estimated 17 million annual deaths [1]. This is approximately 30% of reported annual deaths and is expected to have an increased incidence through the year 2030 [1, 2]. In the USA, there is an estimated total of 27 million individuals afflicted nationwide [1]. As people age, their natural ability to repair and regulate homeostasis of the cardiovascular system declines [3]. This natural decline in cardiovascular health is exacerbated by environmental factors, where clinical intervention will eventually be needed [3, 4]. One of the issues with current therapies for the treatment of cardiovascular disease is that different patients will respond in varying levels to the drugs administered. This discrepancy arises due to unique cellular and genetic conditions underlying these diseases that are specific to the individual. It is for this reason that the concept of personalized medicine has gained attraction in recent years by both physicians and researchers alike. The purposes of this review are to provide some insight into the advancements of stem cell technologies and examine their applications in cardiovascular medicine. In this review, we will discuss different stem cell sources and their current applications, while taking note of the limitations of each. This review takes the stance that induced pluripotent stem cell technology provides an exciting avenue for developing therapeutics with

the aim of making personalized medicine through regenerative therapy a reality.

Embryonic Stem Cells

Brief History of Embryonic Stem Cells

Embryonic stem cells (ESCs) are cells derived from the inner cell mass of the preimplantation blastocyst that retain the ability to differentiate into all three germ layers [5]. Human ESCs have been derived from the human embryo and have displayed pluripotency [6]. Theoretically, ESCs are capable of being expanded in culture indefinitely, which is due to their active telomerase enzymes that prevent telomere shortening, senescence, and rapid apoptosis [7]. These are exciting features of ESCs because it not only allows for the generation of varied cell types for genetic modeling but also for the potential of generating a nearly unlimited supply of cells for use. This aspect makes it particularly attractive for use in cell-based therapies which require large cell numbers. Though human ESCs offered much promise in the years since they were first isolated by Thomson in 1998, public opinion heavily influenced how these cells ultimately could be used [8]. Because the establishment of an ESC line requires the destruction of the developing human embryo, the country was faced with a new ethical dilemma regarding the use of this cell source for research. This national dialogue resulted in a restricted progress in ESC research since there could be no new ESC lines generated with federal funds as declared by the Dickey-Wicker amendment in 1996, which is still in effect to this day. However, subsequent presidential administrations have made suggestions on how to work within the guidelines of Dickey-Wicker while impeding scientific progress as little as possible [9]. Currently, the excess unused eggs from in vitro fertilization (IVF) are the main source of new ESC lines in the USA as they fall into a special category that is permissible under current law [10].

The use of leftover IVF embryos alleviated some of the restrictions ESC research faced previously in that it allowed for a greater number of cell lines with a wide variety of genetic backgrounds to be tested for pathology in disease and necessary pathways in development. Healthy cell lines may also be generated for biobanking-defined human leukocyte antigen (HLA) lines for cell therapies [11]. However, storage protocols must be optimized as current methodologies have led to overall low viability rates for these embryos after thawing [12]. One reason an embryo may not be selected for IVF is that it is deemed to be of low "quality" as determined by the physical characteristics of the embryo that have been associated with low efficacy for implantation in utero [13–15]. Different quality blastocysts have been tested and, surprisingly, are able to give rise to high quality cells that display proper ESC morphology and expression patterns [16, 17]. However, there are some debates regarding the efficiency differences between cell lines in ESC derivation [18, 19]. These cells also show varying capacity to differentiate into cardiomyocytes expressing proper markers regardless of original blastocyst quality [20].

Differentiation of ESCs

The development of protocol for differentiation of ESCs into cardiomyocytes in vitro has been largely based on observations of cardiac development pathways in vivo. These pathways include activities of Wnt, activin/Nodal/TGFβ, BMP, and FGF [21]. A typical protocol may include the following: ESC differentiation into cardiac mesoderm through addition of BMP4, Nodal/activin A, and Wnt/β-catenin; subsequent inhibition of both Wnt/β-catenin and Nodal/activin A to yield cardiac progenitor cells; continued inhibition of Wnt/β-catenin and addition of FGF to induce differentiation into cardiomyocytes; and addition of Wnt/β-catenin, IGF, NRG, FGF1, Notch1, and periostin to support further proliferation [21, 22, 23••, 24].

The generation of cardiomyocytes from ESCs may be a potential therapy for patients that have suffered from myocardial infarction, one occurrence of which can destroy as much as one billion cardiomyocytes [21]. Progress has been made in the development of functional cardiac tissue in vitro. Stevens et al. aimed to create 3D cardiac tissue consisting of ESC-derived cells alone, in a scalable method. To do so, ESCs were cultured and induced to differentiate into cardiomyocytes (through genetic pathways similar to those previously discussed), which were then incubated on a rotating orbital shaker. This yielded an aggregate of disc-shaped cardiac tissue in the center of each plate, which showed synchronous and spontaneous beating. The individual cells were shown to be electrochemically coupled to one another, resembling the functionality that occurs in normal cardiac tissue. This indicates that the experimentally developed cardiomyocytes could be able to functionally integrate with host cardiac tissue [25]. In fact, this functional integration was demonstrated to occur when cardiomyocytes derived from human ESCs were implanted into pig and guinea pig hearts [26, 27]. Cardiomyocyte derived from ESCs has allowed for great advancements in the field of regenerative medicine but is currently limited in their potential for therapies.

Adult Stem Cells

Many tissues retain the ability to regenerate upon damage well into adulthood [28–30]. This regenerative capability is due to somatic stem cells, which retain the ability to differentiate into various cell types of their respective tissues. This is known as multipotency, and while these cells are thought to be more limited in their differentiation potential than ESCs, they do come

with some added benefits. These cells are autologous which reduces the need for immunosuppression for potential therapies. In addition, these cells are derived from an adult individual and thus circumvent the ethical issues surrounding human ESC use.

For cardiovascular regenerative medicine, a candidate autologous stem cell type lies in the bone marrow. Bone marrow-derived stem cells (BMSCs) have been shown to aid in the regeneration of vasculature [31, 32], but their contribution to new cardiomyocyte generation remains controversial [33, 34]. These cells have even been shown to generate perfusable vascular networks for in vitro tissue models [35]. BMSCs have also been used to alleviate limb ischemia. In one study, rats received intravenous injections of bone marrow mesenchymal stem cells. It was found that the BMSCs significantly increased capillary density and improved renal function via an activation of the PI3K-Akt signaling pathway [36]. In another study, the effects of BMSCs in repairing acute ischemic-related renal damage were assessed by injecting Lin(−)Sca-1+ c-Kit+ BMSCs into an area of acute tubular necrosis in mice [37]. The cells differentiated into renal tubular epithelial cells, repairing the ischemic renal tubular injury. Both of these studies illustrated the potential of BMSC therapy in alleviating the effects of ischemia. However, bone marrow extraction is an invasive and painful procedure, and the availability of BMSCs varies from person to person and is further impaired in older or diseased patients. There is little known about the regulatory mechanisms for BMSCs. For these reasons, correcting ischemic damage through BMSC therapy has limited use in clinical applications.

Alternatively, adipose-derived mesenchymal stem cells (ADMSCs) have also been tested for use in cardiovascular regeneration [38–40]. These cells have the benefit of being easily extractable and in ample supply for most patients. Cardiac resident adipose tissue has also been implicated in normal heart repair and homeostasis [39, 41]. ADMSCs have been tested in donors of different health statuses to assess potential functional differences [42]. Because cardiovascular disease is often a consequence of the presence of risk factors such as hypertension and diabetes, ADMSCs must be tested in this context to paint a more accurate picture of their potential as a therapeutic. It was noted that even elderly ADMSCs that are healthy were able to produce functional vascular grafts. However, cells derived from diabetics caused thrombosis in the mouse model [42]. Additionally, the diabetic ADMSCs showed impaired remodeling of the implant due to a decreased capacity for fibrinolysis when compared to healthy ADMSC populations.

Induced Pluripotent Stem Cells

Although human ESCs have been crucial in understanding basic biology surrounding pluripotency and differentiation, current laws restricting their use hinder their efficacy for cell-based therapies. Autologously derived adult stem cells such as BMSCs seem to get around some of the ethical and compatibility issues faced by ESCs but ultimately are limited in that these cells are difficult to isolate or will be in scarce supply for elderly or diseased patients who would be prime candidates for regenerative therapy. An ideal cell source for regenerative medicine needs to be readily available, immunologically compatible, and not ethically dubious to obtain. In 2006, Shinya Yamanaka described successful reprogramming of human somatic cells into a pluripotent state that was similar to ESCs in both its phenotype and transcriptome [43••]. This was accomplished by using retroviral transduction of what have become known as the Yamanaka factors (Oct3/4, c-MYC, Klf4, Sox-2). Yamanaka also demonstrated the differentiation potential of these cells into various lineages via quantification of cell identifying lineage-specific marker expression. The advent of these induced pluripotent stem cells (iPSCs) generated a large interest in the stem cell and regenerative medicine communities because it opened many avenues for research and therapeutics.

Reprogramming of Somatic Cells

The introduction of the exogenous Yamanaka factors into dermal fibroblasts resulted in these cells taking on an ESC-like morphology in that they form rounded colonies. These transcription factors modulate pathways involved in development and metabolism in order to generate and maintain a pluripotent state [44]. Specifically, Oct4, Sox2, and Klf4 were found to regulate developmental pathways while c-Myc regulates metabolism. Subsequent studies show that while c-Myc is dispensable for reprogramming, it does increase the speed at which this process takes place [45]. The exact mechanism of reprogramming is still being actively investigated, although some reports utilizing histone deacetylase inhibitors, or other small molecules that influence chromatin remodeling proteins, have increased the efficiency of reprogramming using only Oct4 and Sox2 overexpression [46, 47]. These findings suggest that chromatin modification is a key component of this process. Elucidating the mechanistic effects of cell reprogramming could help in increasing the efficiency of the overall process. While the genetic mechanisms are being worked out, researchers are adapting methodologies of cell culture and Yamanaka factor introduction to optimize reprogramming protocols.

The original protocol for generating iPSCs relied upon using an integrating viral vector and mouse embryonic fibroblasts (MEF) as feeder cells to aid in reprogramming. Both of these cause significant concerns for translation of these cells into regenerative therapies in human. The presence of xenogenic feeder cells complicates the isolation of human cells, and any residual MEF cells in an implanted tissue could cause unwanted immune reactions and thereby harm the

engrafted construct. In addition, the integrated DNA that supplied the Yamanaka factors could have off-target effects, depending on where it was inserted. To address this, protocols using Matrigel® have been developed for feeder-free culture of iPSCs so as to assure that the final differentiated cells are purely human derived [48]. A few alternatives to integrating viral vector for introducing Yamanaka factors have been studied. Among these are small molecules, ready-made proteins and CRISPR systems for knocking in and out constitutively expressed Yamanaka genes [49–51]. The important theme in these different reprogramming protocols is that they produce iPSCs without integrating vectors. Although these protocols are innovative and may be advantageous over existing protocols, the most efficient way to accomplish this goal currently is by using a nonintegrating Sendai virus as the vector for the reprogramming factors [52, 53]. Using Sendai virus for reprogramming is straightforward, has a high transduction efficiency, and is nonintegrating. These qualities make it ideal for clinically relevant uses such as modeling and treating cardiovascular diseases.

Differentiating into Cardiovascular Lineages

Different stimuli can be employed to differentiate iPSCs into the desired lineage. Included within these parameters will be both biochemical and physical cues, as well as general media components, and each of these components must be optimized for efficient differentiation. Specifically, cardiovascular cell differentiation relies upon temporally controlled activation of the Wnt signaling pathway in order to generate multipotent, brachyury$^+$ mesodermal progenitor cells that can be further differentiated into vascular endothelial cells, vascular smooth muscle cells, or cardiomyocytes [22, 54, 55]. Provision of a small molecule agonist of the Wnt pathway in a temporal-specific manner can markedly improve the generation of various cells of the cardiovascular lineage.

Cardiomyocyte differentiation relies on both up- and downregulation of Wnt signaling. Inhibition of Wnt signaling must be done after they reach the brachyury$^+$ progenitor phase in order to generate high cardiomyocyte yields [22]. This can be accomplished via shRNA knockdown of WNT or small molecule inhibitors [23••, 24]. Utilizing small molecule inhibitors would be better suited for regenerative medicine applications as it does not alter the genome of these cells in any way. Reports of using this method of controlling the Wnt pathway, cultured in insulin and growth factor free medium, have boasted ~90% purity in their differentiated cell populations [23••]. This is a marked increase from the original embryoid body differentiation protocols that would see relatively low cardiomyocyte yields from those populations [56, 57]. Smooth muscle and endothelial cells can be derived from these brachyury$^+$ progenitors without subsequent inhibition of Wnt signaling. These progenitor cells can be cultured on Matrigel® and placed in either commercially available vascular smooth muscle or endothelial cell media, respectively, in order to derive these lineages. Endothelial cells can be further matured into venous or arterial phenotypes using biomimetic bioreactors to provide physical cues in the form of fluid shear stress, which has been shown to have profound effects on vascular endothelial cell behavior [58•, 59, 60]. Differentiated endothelial cells exposed to the fluid shear stress were found to have upregulated vasoprotective markers KLF2 and 4 in addition to upregulating NOTCH signaling that is associated with mature arterial endothelial cells. This type of maturation event will be very important for certain applications of these differentiated cells which will be discussed in the next section.

Applications of iPSCs for Cardiovascular Regenerative Medicine

Modeling Cardiovascular Disease with iPSCs

An attractive feature of iPSCs is that they retain all genetic traits of the cell donor, and this can be leveraged to model diseases in vitro. This method is advantageous, compared to using somatic myocardium, because iPSCs have a larger replicative capacity and are much more readily available than donor heart tissue. iPSC technology has been used to study hypertrophic cardiomyopathy (HCM) where cellular phenotype was shown to be conserved in iPSC differentiated cardiomyocytes [61]. This model was the first to show that RAS signaling was perturbed in human cardiomyocytes with HCM causing mutation in *PTPN11*. Other genetic backgrounds of HCM have been assessed using iPSC to gain insights into the diseases' electrical alterations [62, 63••]. These studies showed that high calcium concentration during diastole causes a progression of HCM phenotype in cardiomyocytes with a mutated MYH7. Human iPSC-based disease modeling provided a unique perspective over mouse models since the structure of sarcomeric proteins between mouse and human is quite different [64]. These differences cause alternate cellular phenotypes in mouse and human like sarcomeric backgrounds with the same mutation [65].

Vascular diseases such as supravalvular aortic stenosis (SVAS) can also be modeled with hiPSC technology [66]. SVAS is characterized by haplodeficiency of elastin and eventually the reduction in luminal diameter due to proliferating vascular smooth muscle cells in the supravalvular aortic region. This phenotype was recapitulated using iPSC-derived smooth muscle cells in that they were noted to have an increased proliferation rate as compared to wild-type cells. The iPSC modeling system also revealed a disorganization of contractile bundles in SVAS smooth muscle cells, indicating that the cellular phenotype is particularly robust in terms of

modeling SVAS. iPSC modeling systems offer a dual capacity for investigators in that they cannot only offer a platform for gaining valuable insights into the mechanisms of disease progression but can also be used as a tool for drug screening to treat the disease [67]. Drug screens on human cells will give a better picture of how human cells will react to treatment than using mouse models. This concept may also be applied in the clinic as a means of personalized medicine to determine what drugs are the most effective for an individual's cells [68, 69, 70••].

Tissue Engineering and Regenerative Therapies

Stem cells also offer a great platform for developing clinical interventions to treat cardiovascular disease. A few examples currently in the clinical trial stage are listed in Table 1. The goal of regenerative medicine is to replace or restore nonfunctional tissues. A very exciting, albeit ambitious, approach is to generate entire hearts for transplant patients [71, 72]. This methodology aims to address two major issues transplant recipients face: the lack of availability of organs and complications due to immune rejection. The perfusion decellularization method seeks to remove all endogenous cells within a mature adult heart while leaving behind the extracellular matrix that keeps the organ geometry and vascular structure intact. This allows investigators to utilize xenogenic organs of similar size to help fill the clinical demand. Different methodologies for recellularizing the extracellular matrix (ECM) scaffold have been explored where it was noted that re-endothelialization prior to parenchymal recellularization increased cell infiltration and ventricular functionality [72]. Human iPSC-derived cardiac progenitors have been used to repopulate mouse heart ECM scaffolds. This strategy resulted in proper endothelial and myocardial differentiation and localization [73]. However, arrhythmic beating and poor contractile force were noted in the construct. This implies that, although the ECM can direct proper differentiation of progenitors, the tissue may not be maturing properly. This could be due to missing mechanical or electrical cues that are necessary for functional tissue maturation and myocyte coupling. Additionally, other cell types may be necessary for maturation of functional heart tissue such as cardiac fibroblasts [74, 75]. More basic biology of its constituent components must be understood in terms of how to generate physiologically relevant vasculature, myocardium, and pacemakers before the dream of generating a fully functional heart can be realized.

Each of these components of healthy heart tissue can be generated individually in order to provide insights into the mechanisms that facilitate proper development and to potentially provide therapeutic interventions at a smaller scale. Tissue-engineered vascular grafts (TEVG) aim to provide fully functional vasculature for patients in need of a bypass surgery [76••]. Methods have been attempted to use artificial scaffolds of different porosity to generate vessels in situ [77–79]. These grafts showed infiltration of native endothelial and vascular smooth muscle cells. Although there was some remodeling and ECM deposition, the artificial polylactic acid scaffolds remained largely intact after 12 months. It was noted that these acellular scaffolds showed macrophage infiltration, which has positive implications for vascular remodeling by influencing vascular cell behavior and ECM deposition [80–82]. In addition, these scaffolds also need to be preseeded with autologous bone marrow-derived endothelial cells. Ideally, a TEVG would utilize a cell source that is less

Table 1 Cardiovascular stem cell therapies in the USA

Disease	Type	Status	Time frame	Cell source	Phase	Delivery mechanism	Clinical trial ID
Ischemic cardiomyopathy	Interventional	Completed	September 12, 2005–June 05, 2015	Autologous BMSC	1	Intramyocardial injection	NCT00203203
Chronic ischemic left ventricular dysfunction	Interventional	Ongoing	December 02, 2013–	Allogenic hMSC	2	Transendocardial injection	NCT02013674
General aging	Interventional	Ongoing	July 18, 2016–	Allogenic hMSC	2	Intravenous injection	NCT02065245
Coronary artery disease	Interventional	Ongoing	April 10, 2006–	Autologous BMSC-derived aldehyde dehygrogenase bright cells	1	Intramyocardial injection	NCT00314366
Ischemic cardiomyopathy	Interventional	Completed	May 15, 2007–October 15, 2014	Autologous c-kit+ cardiac stem cells	1	Intracoronary injection	NCT00474461
Peripheral arterial disease	Interventional	Recruiting	April 25, 2016–	Autologous ADSC	1	Intravenous injection or intramuscular injection	NCT02756884
Congenital heart disease	Interventional	Ongoing	January 29, 2008–	BMSC	2	Cardiomyoplasty	NCT01034007
Myocardial infarction	Interventional	Completed	July 2014–April 2016	Allogenic mesenchymal bone marrow cells	3	Intravenous injection	NCT02672267
Acute myocardial infarction	Interventional	Ongoing	June 2014–	Allogenic cardiac stem cells	2	Intracoronary infusion	NCT02439398
Cardiomyopathy	Observational	Recruiting	June 2014–	hiPSC	–	–	NCT02417311
Chronic myocardial ischemia	Interventional	Recruiting	January 2016–	Autologous MSC	2	Intramyocardial injection	NCT02462330
Cell type			No. of trials	% Observational		% Interventional	
hiPSC			5	100		0	
ADSC/BMSC			41	4.9		95.1	

Examples of recently completed or ongoing stem cell-based therapy trials. Total trials listed on clinicaltrials.gov for cardiovascular diseases are given for each cell type. Currently, there are no clinical trials using embryonic stem cells in the USA, so this category was omitted from this table

invasive to collect. Some labs are utilizing iPSC technology to develop engineered blood vessels. Two-millimeter-thick polyglycolic acid (PGA) scaffolds have been utilized to develop TEVG by seeding them with vascular smooth muscle cells (VSMC) [76••, 83]. Static culture for approximately 8 weeks on this scaffold yielded an implantable vessel with inherent ECM composition and is perfusable in vivo. These vessels do not harbor sufficient strength to withstand burst pressure which may be due to lack of sufficient collagen deposition and/or suboptimal remodeling efficiency. These vessels also must be coated with endothelial cells to prevent thrombogenicity and be able to interact with circulating leukocytes. Endothelial specification must be taken into account as arterial endothelium behaves much differently than venous [84, 85]. Endothelial cells contain robust mechanical sensing mechanisms that influence gene expression and remodeling, and these abilities have been leveraged to specify differentiation into an arterial-like phenotype via biomimetic luminal flow [58•, 86].

Cardiac patches offer a great therapeutic to aid repair after myocardial infarction by reducing scar tissue formation and global remodeling [87–89]. Similar to what is seen in whole organ engineering attempts, poor electrical coupling of cells is seen within cardiac patches [90]. The lack of electrical coupling of these cells is a recurring issue that researchers are attempting to circumvent via biomaterial and cell maturational interventions. Nanostructural modifications of the scaffolds used in generating these tissues have provided improvements in tissue morphology and electrical coupling of mature cardiomyocytes [90–92]. Additionally, biomaterial modifications aimed at mimicking natural ECM protein and polysaccharide composition have been developed to aid progenitor cell differentiation into cardiomyocytes [93]. The modified hyaluronic acid patches showed an increased expression of cardiac markers, including the gap junction protein connexin43, as compared to other synthetic scaffolds. However, testing of the signal propagation characteristics of these differentiated cardiac cells is needed to confirm if this expression is sufficient to allow synchronous contraction with native myocardium. As an alternative approach, cardiac patches may also be used as a mode of delivery for cardiac progenitor cells (CPC) [94]. In this way, the functionality of the patch tissue itself is not a factor because the progenitor cells will translocate from the pericardium into the infarct site where they will mediate repair.

Stem cells may also be used to generate biological pacemakers, which is a crucial development for whole organs to be viable for translation. The sinoatrial node (SAN) is thought to be the initiator of the electrical signal that controls beating [95]. Current strategies for generating biological pacemakers aim to functionally mimic nodal cells of the SAN. Human mesenchymal stem cells have been transduced to express the

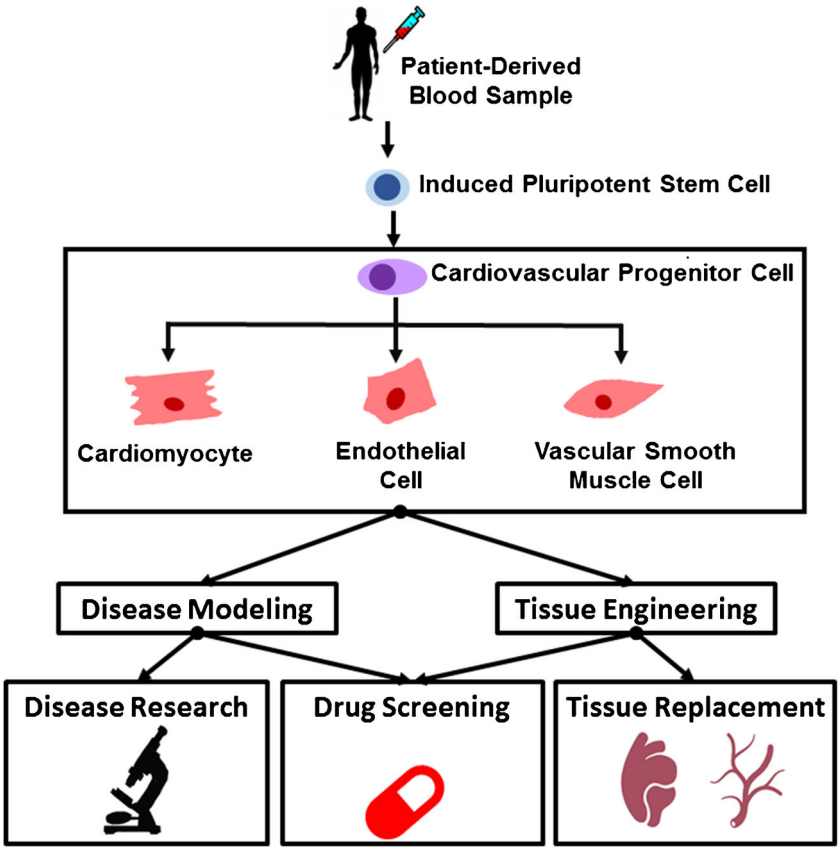


Fig. 1 Schematic of stem cell applications in cardiovascular medicine. Schematic depiction of iPSCs derived from peripheral blood that are differentiated into a cardiovascular lineage. These cells can be utilized for either tissue engineering constructs to replace dysfunctional vasculature or myocardium for clinical interventions or drug screening in complex, 3D tissues. 2D cell culture also allows for modeling of disease with these cells and high-throughput drug screening

pacemaker-specific HCN2 ion channel and subsequently expressed cardiac gap junction proteins [96, 97]. It is important to note that these cells are not pacemakers within themselves but rather provide a mechanism of depolarization for the resident cardiomyocytes coupled to the $HCN2^+$ hMSC via the I_f current generated by HCN2. This multicell setup initiated subsequent action potential firing when resident cardiomyocytes became hyperpolarized between beats in a canine model [98], although it is unclear if pacing was due entirely to implanted cells. Researchers have attempted to isolate nodal-like cells from the heterogeneous mixture of hESC-derived cardiomyocytes but ultimately have been unable to show their ability to regulate sustained contraction in vivo [99, 100, 101••]. Transcription factors Shox2 and Tbx18 have each been implicated in the maintenance of cardiac pacing in nodal cells and have been used to generate autonomous pacemakers from mouse stem cells [102, 103]. Although these cells were found to have pacing capabilities, they still must be further refined to fully recapitulate physiologic pacing and be shown to maintain pacing functionality long term.

Conclusion

The ability to generate cells of the cardiovascular system allows for more relevant in vitro disease modeling systems and designing of tissue-engineered therapeutics. Various cell sources have been assessed for generating cardiac, endothelial, and smooth muscle cells with robust phenotypes. Studies with embryonic stem cells offered great insights into the methodologies that can reproducibly differentiate into these lineages although their use was limited due to political and ethical concerns of the public. Autologous adult stem cell sources are very attractive, from a therapeutics perspective, due to their inherent immune compatibility with the host. However, they are not always abundant or the most accessible. In cases where the cells can be extracted, there are still issues with the maintenance of these cells due to the lack of knowledge of the basic biology for BMSCs. iPSCs represent the most versatile stem cells in that they have the combined attributes of being autologous, easily accessible, and not ethically dubious. These qualities make them a prime candidate for both experimental studies in different genetic backgrounds and engineering therapies as outlined in Fig. 1. Reprogramming and differentiation to a specific cell lineage requires time, so interventions using this cell source would be limited to nonurgent treatments as patients would need to wait for weeks for the tissue to mature. Still, stem cells show great promise in recapitulating the physiology and architecture of complex tissues, though more fine-tuning of the differentiation protocol is necessary to become more efficient in cardiomyocyte generation. In order to accomplish this goal, more of the basic biology of stem cells must be pursued so that these therapeutics approaches can become more robust. An interdisciplinary approach is necessary to accomplish the goal of personalized regenerative medicine. Collaborations between stem cell biologists, bioengineers, and material scientists will offer the most efficient path to translation for these projects. The expertise from these fields will allow researchers to discern and recapitulate the proper cell environments to allow for the maturation of these tissues that will yield in physiologically relevant implants.

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Compliance with Ethical Standards

Conflict of Interest Christopher W. Anderson, Nicole Boardman, Jiesi Luo, Jinkyu Park, and Yibing Qyang declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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